RESEARCH PROPOSAL

THERAPEUTIC POTENTIAL OF BERBERINE- POLYMER CONJUGATED NANOPARTICLES AGAINST SKIN CANCER

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Submitted by

Monisha Singhal
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Supervisor
Dr. Nidhi Gupta
Senior Research Scientist

Co- supervisor
Dr. Sreemoyee Chatterjee
Associate Professor

Department of Biotechnology

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Introduction

Skin cancer is a tumor or abnormal growth of cells in the skin. The primary cause of skin cancer is over exposure to sunlight. However, there are other factors that may lead to skin cancer such as repeated exposure to X-ray radiation, certain chemical toxins and a family history of skin cancer. According to World Health Organization (WHO), the incidences of skin cancer had been increased over the past few decades. It has been estimated that between 2 and 3 million non- melanoma skin cancers and 132,000 melanoma skin cancer occur each year globally. Also, skin cancers are the most common human cancers as one in every three cancer is a skin cancer. The types of skin cancer is determined after the type of skin cell from which they arise. Broadly it is divided into Non-melanoma: Basal cell cancer (BCC) and Squamous cell cancer (SCC), and Melanoma skin cancer. BCC is the most common cutaneous malignancy contributing 65–75% of all skin cancers, globally. Also there is a significant difference between the incidences of skin cancer in Asians and Blacks as compared to the Caucasians (1). Non- melanoma skin cancers (NMSCs) comprise of 1%-2% of cutaneous neoplasms in Indians, in contrast to one‐third in whites. Such a low rate of NMSCs in dark skin population is due to protection offered by melanin pigment against ultraviolet radiation (UVR) -induced DNA damage. The statistics shows that SCC represents 30%-65% of skin cancers in blacks and Indians and 15%-25% in whites, whereas BCC contributes to 65%-75% of skin cancers in whites and 20%-30% in Asian Indians (2). In India, out of all the cancers diagnosed less than 1% is skin cancer with SCC to be most prevalent one. Although, when compared to western world the incidence of skin cancers in India is lower but due to larger population base the absolute number of cases is estimated to be significant (3).

The conventional method for the treatment of skin cancer is excision biopsy. Other treatments include: curettage and diathermy, liquid nitrogen, imiquimod or 5-fluorouracil (5-FU), radiotherapy or excision, and flap repair/graft. Topical imiquimod therapy appears to provide relatively low cure rates for melanomas. IFN-α treatment may be employed in patients with stage II and III melanoma as an adjuvant therapy. For some metastatic patients, systemic chemotherapy (dacarbazine, temozolomide, or carboplatin/paclitaxel) continues to play an important role.

Many bioactive compounds derived from different plants have been used for hundreds of years for the treatment of countless diseases. In context to this, a natural compound called Berberine (BBR) has been extensively used against variety of cancers. Berberine is a quaternary benzylisoquinoline alkaloid from the class of protoberberines found in the roots, stem and bark of *Berberis (Berberis vulgaris, Berberis croatica L., Berberis aquifolium, Coptis (Coptis chinensis L., Coptis japonica L.), and Hydrastis (Hydrastis Canadensis)*. It has been used in Chinese, Unani, and Indian Ayurveda medicine since ancient times (4, 5). It has been proved that the compound have anticancer effects on various tumor cell lines like colon, breast, prostate, oral glioblastoma, hepatoma, melanoma, colorectal, lung and leukemia.

Berberine: Mechanism of action

The DNA-binding potential of Berberine was first postulated by Krey and Han. The most important factor for the anticancer activity of the compound is its strong affinity with the DNA molecule as Berberine is a positively charged molecule due to the presence of nitrogen at 7^{th} position while on the contrary DNA is a negatively charged molecule which upon reaction binds efficiently. Simultaneously, enzyme inhibition activity like telomerase inhibition has been seen which may also contribute to its anticancer activity (6). As a results of topoisomerase inhibition, the compound also possess antiproliferative activity (7).

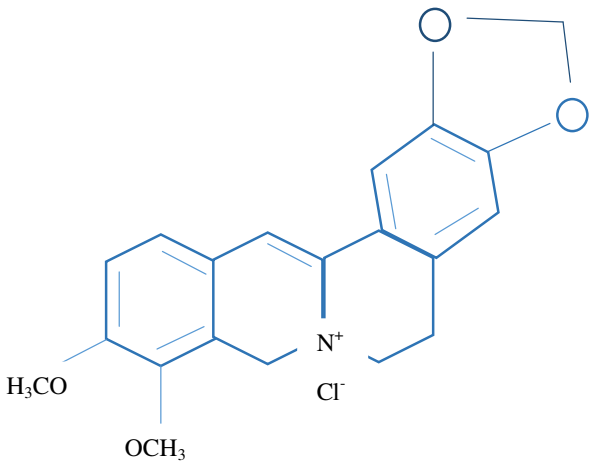


Figure 1: Structure of Berberine

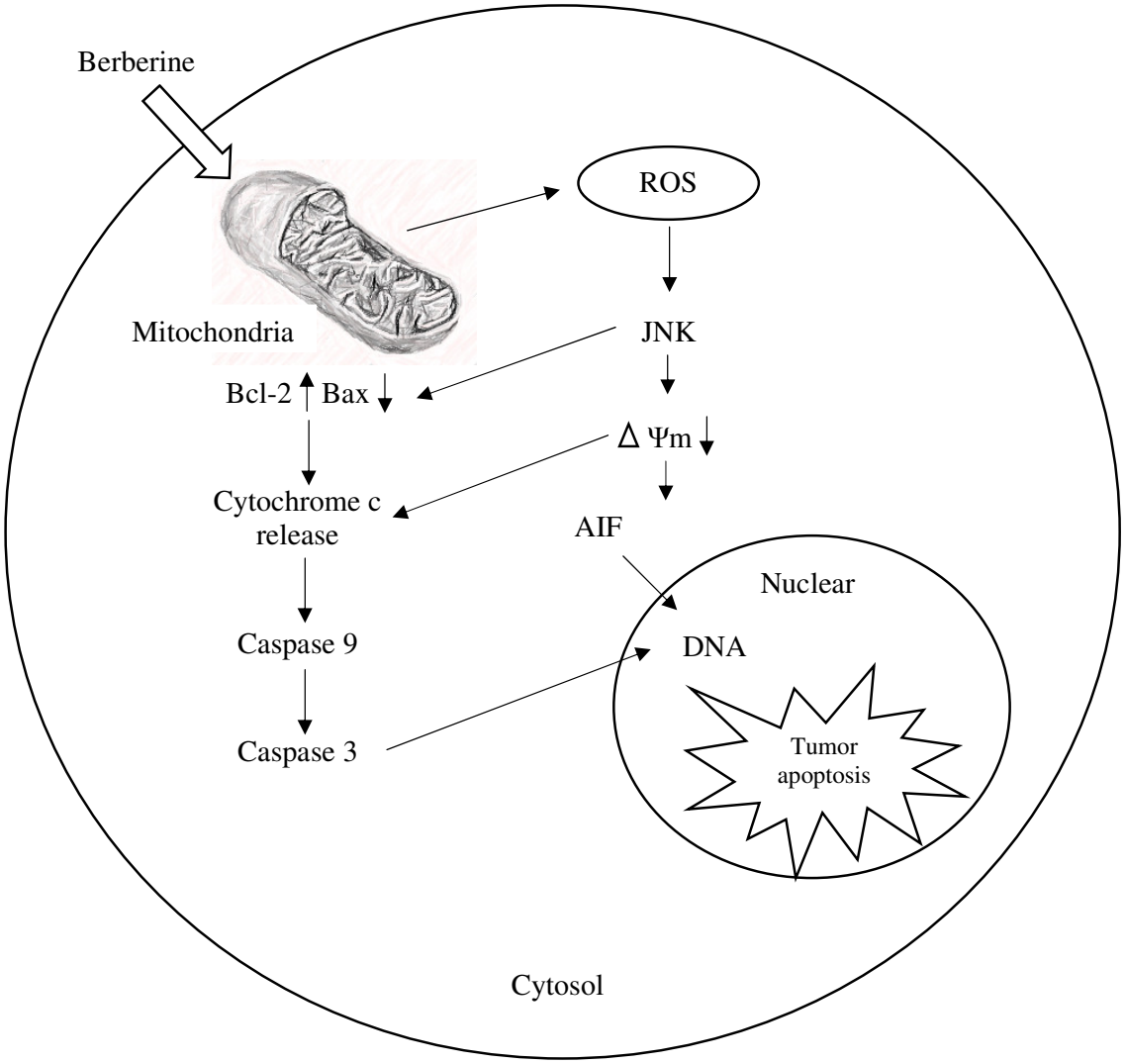


Figure: 2 Mechanisms of berberine-exerted antitumor activity.

In mitochondria, berberine increases the production of reactive oxygen species (ROS), which then activated the pro-apoptotic JNK signaling. This phosphorylated JNK triggers mitochondria membrane potential (ΔΨm) depolarization and downregulation of anti-apoptotic protein and Bcl-2 together with the upregulation of proapoptotic protein called Bax. Downregulation of anti-apoptotic Bcl-2 family protein simulteneously with loss of ΔΨm, leads to increase in the release of cytochrome c and apoptosis inducing factor (AIF) from mitochondria which eventually triggered the caspase-dependent and caspase-independent (8).

Review of literature

Anticancer activity of BBR in different cancers

Piyanuch *et. al*., studied the role of berberine in human colorectal cancer cells and found that berberine induces cell growth arrest and apoptosis in human colorectal cancer cells. Also, NAG-1 and ATF3 expression plays a crucial role in berberine-induced apoptosis (9). A study was carried out on breast cancer cells (MCF-7) to determine the effect of berberine and it was observed apoptosis in MCF-7 breast cancer cells was induced by Berberine through a mitochondria-dependent pathway. This had been achieved by increasing the levels of cytoplasmic cytochrome c, caspase-9 activity and cleavage of PARP, simultaneously decreasing the levels of Bcl-2 (10). Similar study was carried out on two breast cancer cell lines- MCF-7 and MDA-MB-231 and it was found that the alkaloid berberine inhibits the growth of Anoikis-resistant MCF-7 and MDA-MB-231 breast cancer cell lines by inducing cell cycle arrest. Also, when compared with doxorubicine treatment, Berberine inhibit growth to greater extent (11). Murthy *et. al*., studied the effect of berberine targeting multiple pathways to induce cell death in cultured human colon cancer cells. They found that proliferation of cancer cells was inhibited in dose- and time- dependent manner with cell cycle arrested at G2/M phase supported by induction of p21 expression. Also, the compound showed minimum toxicity in normal cells (12). Refaat and his co-workers studied the effect of berberine on TRAIL‐induced antitumor *in vitro* using recombinant TRAIL and *in vivo* using a 4T1 murine breast cancer model with a combination of anti‐DR5 (death‐inducing TRAIL receptor) monoclonal antibody therapy. Combination of Berberine with TRAIL enhance apoptosis and may become a potential new adjuvant to overcome the resistance of cancer cells to TRAIL/DR5‐mediated therapy (13). Combinational study of drug namely tamoxifen and berberine was done by Wen and his co- workers on drug‐sensitive MCF‐7 and drug‐resistant MCF‐7/TAM cells. Results showed that the combination of berberine and tamoxifen induce cell growth inhibition more efficiently than tamoxifen alone. This combination was more effective to arrest the cell growth at G1 phase as compared with tamoxifen alone (14). Zhao *et. al.,* showed that BBR induces Double Strand Breaks (DBS) with increase in the release of cytochrome c which eventually trigger the Caspase 9-dependent apoptosis. They studied the effect of BBR on growth of triple negative breast cancer cell (TNBC) lines (MDA-MB-231 and BT549), in -vitro and in- vivo (15). From 2017 onwards, advance studies has been done on berberine related with chemoresistence, its

efficiency with in combination with other drugs or genes involved in tumor suppression or cell proliferation. Pan *et. al*., cultured MCF-7 breast cancer cells under hypoxia to make cells resistance to doxorubincin (DOX) and then different doses of Berberine on these cells was given. As a results of different doses, Low-dose berberine shows that DOX chemosensitivity in MCF-7/hypoxia cell could be resensitized, however, high-dose could directly induced apoptosis (16). One step DMBA induced breast cancer in female Sprague Dawley rats was studied by Karnam and his co- workers. They also found significant effects of the compound (17). The first study on combined effects of berberine and cinnamaldehyde *in- vitro on* A549 cells showed that both induce apoptosis and prevent cell proliferation. This combination reduces the nutrient uptake via AMPK-reduced AQP-1 expression, which ultimately results in starvation of tumor cells (18). The metastasis of triple negative breast cancer (TNBC) cells was also been studied. The inhibitory effects of berberine on tumor growth was measured by the levels of MMP-2, MMP-9 and TGF-β1. It was found that BBR decrease the level of MMP-2 expression as well as cell migration in TNBC cells. In breast cancer xenograft models- MDA-MB231 and 4T1, tumor growth and lung metastasis were effectively decreases by BBR treatment (19). The mechanism by which mechanism by which BBR either alone or combining with metformin inhibit lung cancer cell proliferation was determined by Zeng and his co-workers. They suggest that BBR inhibits growth, migration and invasion of lung cancer cells by inhibiting SP1 and PDPK1 which results in the inhibition of DNMT1 gene expression (20). The application of nanotechnology in the field of medicine plays an important role. Nanoparticles has been used to deliver drugs in various diseases most importantly in cancers. A conjugated nanoparticles i.e. BBR-AgNPs with polyethylene glycol-functionalized folic acid (FA-PEG) was developed to induce apoptosis in breast cancer cells. The conjugated nanoparticle could efficiently release BBR molecules into the specific cancer site. As a result there was an increase in apoptotic induction and condensed nuclei in cancer cells (21).

Nanoparticle synthesis using chitosan

The second most abundant natural bio-polymer after cellulose is Chitosan. It is a linear polyaminosaccharide obtained by N-deacetylation of Chitin, found in fungal cell wall or shell of crustaceans. It is biocompatible and biodegradable and has been extensively used in the preparation of nanoparticles. Because of these properties it become a suitable carrier for various therapeutic agents like peptides, DNA/RNA, proteins or drugs. Katas *et.al*., developed chitosan nanoparticles using dextran sulphate as a cross linking agent. They

entrapped bovine serum albumin and siRNA to determine the entrapment efficiency and found that the entrapment efficiency was proportional to the ratio of weight of chitosan and dextran sulphate (24). Ghadi *et. al*., explored the biomedical and pharmaceutical applications of novel magnetic chitosan nanoparticle (25). A comparative analysis for chitosan nanoparticle synthesis has been done using three different cross linking agents- Tripolyphosphate (TPP), Dextran Sulphate (DS) and poly- D-glutamic acid (PGA), loaded with siRNA. Among the three, TPP produced the smallest particles having high entrapment and binding efficiencies. Also, it was found to be most stable system, resulting in slow release of siRNA (22). Mohammed *et. al*., synthesized a nanoparticle for drug delivery due to its biodegradable capability. They prepared a nanoparticle with positive surface charge and possess adhesive properties to adhere mucus membranes and release the drug (23). A Chitosan Nanoparticles (CNP) was synthesised via ionic gelation using tripolyphosphate (TPP) as a cross-linker to deliver glutamic acid, fluorescently-labeled with fluorescein isothiocyanate to track *in vitro*. The target was achieved as fGA was released from CNP, accumulated into cells as early as 6 hours post-treatment (26).

A conjugated hyaluronic acid- chitosan based nanoparticles entrapping everolimus, a key molecule in allograft organ rejection prevention, was synthesied to treat Bronchiolitis obliterans syndrome (BOS). Hyaluronic acid was used to target CD44 present on mesenchymal cells of lungs. Results suggest that this formulation could boost its internalization to releases the drug more efficiently (27).

Hypothesis

Berberine is a natural compound which possess anticancer activity against different types of cancer cells. Based upon that we can hypothesized that it may also have some potential against skin cancer.

Objectives

The objectives of this study are –

- Designing and synthesis of Berberine polymer conjugated nanoparticles
- Characterization of conjugated nanoparticles
- *In vitro* cytotoxicity assay on human embryonic kidney (HEK 293) cell line
- *In vitro* anticancer effect of Berberine chitosan conjugated nanoparticles (BCCN) on skin cancer cell line, namely A431
- *In vivo* investigation of therapeutic potential of conjugated nanoparticles against skin cancer in mice model

Methodology

Preparation of conjugated nanoparticles

The nanoparticles shall be prepared using chitosan, a derivative of chitin obtain from fungal cell wall or shells of crustaceans.

Characterization of conjugated nanoparticles

Dynamic Light Scattering (DLS): a technique in physics, shall be used to determine the size, size distribution and surface charge of the nanoparticles.

UV-Vis Spectra: Analysis will be done by using UV-Vis spectrophotometer. The releasing efficiency of Berberine from the synthesised nanoparticle shall be done using this technique.

Scanning Electron Microscope (SEM)/ Transmission Electron Microscope (TEM): these techniques shall be used to determine the size and shape of the nanoparticles.

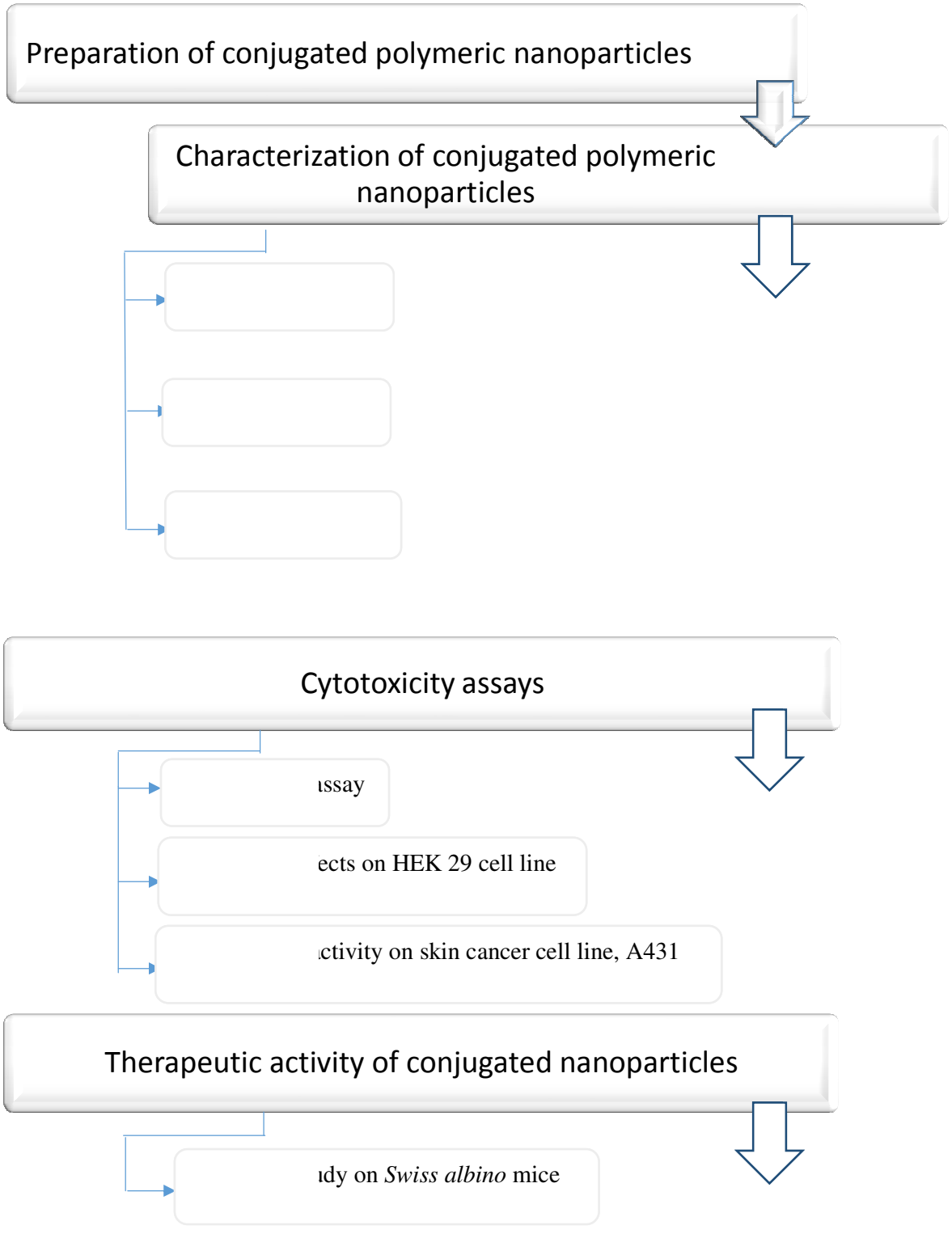
In- vitro cytotoxicity assays

Non-cancerous cell line, Human Embryonic Kidney 29 cell line shall be used to determine the toxic effects of conjugated nanoparticles. Tumor cell, namely A431 skin cancer cell line shall be used to determine anticancer effects of the conjugated nanoparticles.

Therapeutic activity of conjugated nanoparticles

Swiss albino mice shall be used to study the potential therapeutic effects of Berberine chitosan conjugated nanoparticles against skin cancer. The two stage cancer induce method using Dimethylbenzanthracene (DMBA) and Tetradeconyl phorbol-13-acetate (TPA) shall be used to induce skin cancer in mice models.

Plan of work


Preparation of conjugated polymeric nanoparticles
Characterization of conjugated polymeric nanoparticles
Cytotoxicity assays
ıssay
ects on HEK 29 cell line
ctivity on skin cancer cell line, A431
Therapeutic activity of conjugated nanoparticles
ıdy on *Swiss albino* mice

Group 1

- Control, DMBA+ TPA

Group 2

- Positive control, CNPs

Group 3

- Test, DMBA+TPA+ CNPs

Group 4

- Test, DMBA+TPA+ CNPs

Group 5

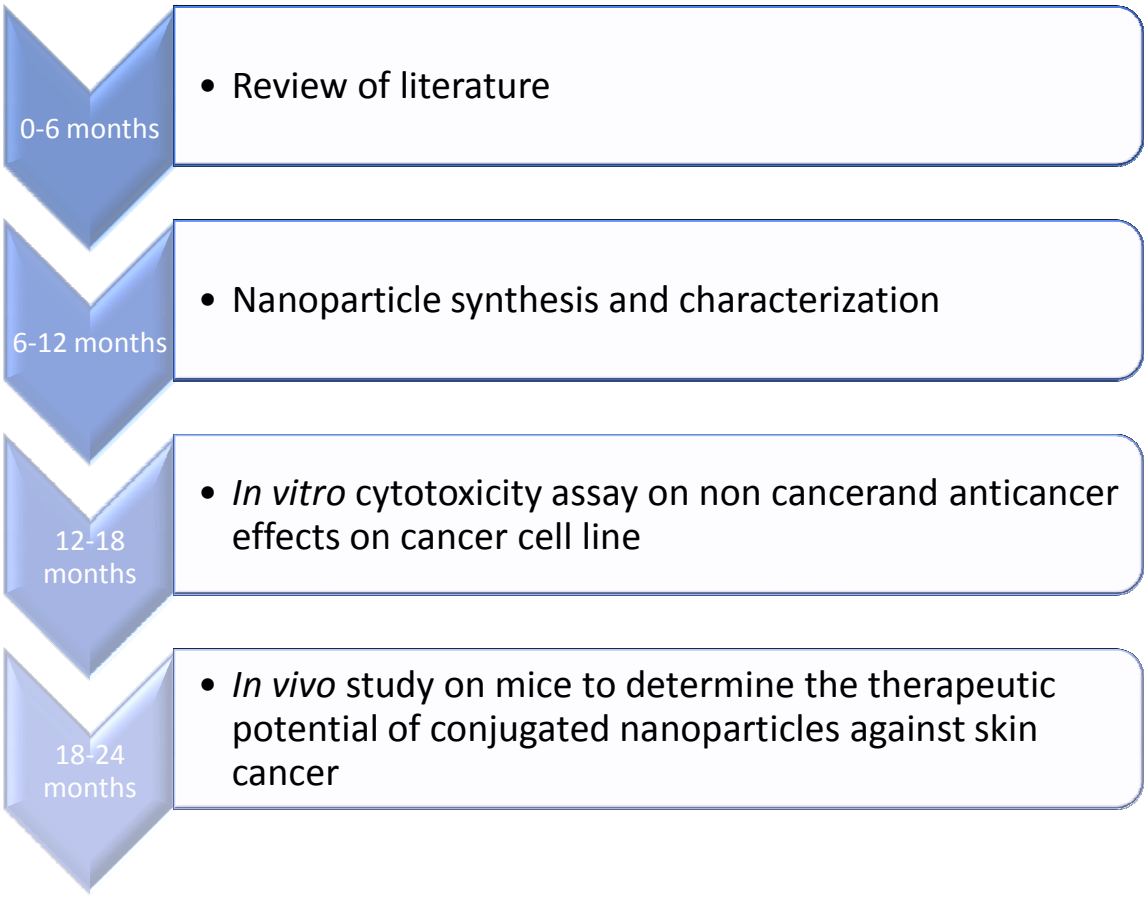
- Test, DMBA+TPA+ CNPs

Group 5A

- Test, DMBA+TPA+ Berberine

Group 5B

- Test, DMBA+ CNPs


0-6 months
• Review of literature
6-12 months
• Nanoparticle synthesis and characterization
12-18 months
• *In vitro* cytotoxicity assay on non cancerand anticancer effects on cancer cell line
18-24 months
• *In vivo* study on mice to determine the therapeutic potential of conjugated nanoparticles against skin cancer

References

1. Kumar, S., Mahajan, B.B., Kaur, S., Yadav, A., Singh, N. and Singh, A. (2014). A Study of Basal Cell Carcinoma in South Asians for Risk Factor and Clinicopathological Characterization: A Hospital Based Study. Journal of Skin Cancer, **9**.
2. Khullar, G., Saikia, U.N., De, D. and Radotra, B. D. (2014). Nonmelanoma skin cancers: An Indian perspective. Indian Journal of Dermatopathology and Diagnostic Dermatology, **1**: (2).
3. Lal, S.T., Banipal, R.P.S., Bhatt, D.J. and Yadav, H.P. (2016). Changing Trends of Skin Cancer: A Tertiary Care Hospital Study in Malwa Region of Punjab. Journal of Clinical and Diagnostic Research, **10**: (6).
4. Chen, W., Miao, Y.Q., Fan, D.J., Yang, S. S., Lin, X., Meng, L. K. and Tang, X. (2011). Bioavailability Study of Berberine and the Enhancing Effects of TPGS on Intestinal Absorption in Rats. *AAPS PharmSciTech,*, **12**; 2.
5. Patil, J. B., Kim, J. and Jayaprakasha, G. K. (2010). Berberine induces apoptosis in breast cancer cells (MCF-7) through mitochondrial-dependent pathway. European Journal of Pharmacology **645**; 70–78.
6. Bhadra, K. and Kumar, G. S. (2010). Therapeutic potential of nucleic acid-binding isoquinoline alkaloids: binding aspects and implications for drug design. Med. Res. Rev., **31**; 821-862.
7. Tan, W., Li, Y., Chen, M. and Wang, Y. (2011). Berberine hydrochloride: anticancer activity and nanoparticulate delivery system. International Journal of Nanomedicine, **6**; 1773–1777.
8. Xie, J., Xu, Y., Huang, X., Chen, Y., Fu, J., Xi, M., & Wang, L. (2014). Berberine-induced apoptosis in human breast cancer cells is mediated by reactive oxygen species generation and mitochondrial-related apoptotic pathway. Tumor Biology, **36**(2), 1279–1288.
9. Piyanuch, R., Sukhthankar, M. and Baek, S.J. (2007). Berberine, a natural isoquinoline alkaloid, induces NAG-1 and ATF3 expression in human colorectal cancer cells. Cancer Lett., **258** (2): 230–240.
10. Patil, J.B., Kim, J. Jayaprakasha, G.K. (2010). Berberine induces apoptosis in breast cancer cells (MCF-7) through mitochondrial-dependent pathway. European Journal of Pharmacology **645**; 70–78.

11. Kim, J.B., Yu, J-H., Koa, E. Lee, K.-W., Song, A.K., Park, S.Y., Shin, I., Han, W. and Noh, D.Y. (2010). The alkaloid Berberine inhibits the growth of Anoikis-resistant MCF-7 and MDA-MB-231 breast cancer cell lines by inducing cell cycle arrest. Phytomedicine, **17**; 436–440.
12. Murthy, K.N.C., Jayaprakasha, G.K. and Patil, B.S. (2012). The natural alkaloid berberine targets multiple pathways to induce cell death in cultured human colon cancer cells. European Journal of Pharmacology, **688**; 14–21.
13. Refaat, A., Abdelhamed, S., Yagita, H., Inoue, H., Yokoyama, S., Hayakawa, Y. and Saiki, I. (2013). Berberine enhances tumor necrosis factor‐related apoptosis‐inducing ligand‐mediated apoptosis in breast cancer. Oncology Letters, **6**; 840-844.
14. Wen, C., Wu, L., Fu, L., Zhang, X. and Zhou, H. (2016). Berberine enhances the anti‐tumor activity of tamoxifen in drug‐sensitive MCF‐7 and drug‐resistant MCF‐7/TAM cells. Molecular Medicine Reports **14**; 2250-2256.
15. Zhaoa, Y., Jingb, Z., Lva, J., Zhanga, Z., Lina, J., Caoa, X., Zhaoa, Z., Liuc, P. Maoa, W. (2017). Berberine activates caspase-9/cytochrome c-mediated apoptosis to suppress triple-negative breast cancer cells in vitro and in vivo. Biomedicine & Pharmacotherapy **95**; 18–24.
16. Pan, Y., Shao, D., Zhao, Y., Zhang, F., Zheng, X., Tan, Y., He, K., Li, J. and Chen, L. (2017). Berberine Reverses Hypoxia-induced Chemoresistance in Breast Cancer through the Inhibition of AMPKHIF-1α. International Journal of Biological Sciences, **13** (6); 794-803.
17. Karnama, K.C., Ellutlaa, M., Boddulurua, L.N., Kasalaa, E.R., Uppulapua, S.K., Kalyankumarrajua, M. and Lahkar, M. (2017). Preventive effect of berberine against DMBA-induced breast cancer in female Sprague Dawley rats. Biomedicine & Pharmacotherapy **92**; 207–214.
18. Meng, M., Geng, S., Du, Z., Jingjing Yao, J., Zheng, Y., Li, Z., Zhang, Z., Li, J., Duan, Y. and Du, G. (2017). Berberine and cinnamaldehyde together prevent lung carcinogenesis Oncotarget, **8** (44); 76385-76397.
19. Kima, S., Leed, J., Youc, D., Jeongc, Y., Jeone, M., Yua, J., Kima, S.W., Nama, S.J. and Lee, J. E. (2018). Berberine Suppresses Cell Motility through Downregulation of TGF-β1 in Triple Negative Breast Cancer Cells. Cell Physiol Biochem, **45**; 795-807.

20. Zheng, F., Wu, J., Tang, Q., Xiao, Q., Wu, W.Y. and Hann, S.S. (2018). The enhancement of combination of berberine and metformin in inhibition of DNMT1 gene expression through interplay of SP1 and PDPK1. J. Cell. Mol. Med., **22** (1); 600-612.
21. Bhanumathi, R., Manivannan, M., Thangaraj, R. and Kannan, S. (2018). Drug-Carrying Capacity and Anticancer Effect of the Folic Acid- and Berberine-Loaded Silver Nanomaterial to Regulate the AKT-ERK Pathway in Breast Cancer. ACS Omega, **3**; 8317−8328.
22. Ghadi, A., Mahjoub, S., Tabandeh, F. and Talebnia, F. (2014). Synthesis and optimization of Chitosan nanoparticles: Potential applications in nanomedicine and biomedical engineering Iran. Caspian J Intern Med, **5** (3); 156-161.
23. Mohammed, M.A., Syeda, J.T.M., Wasan, K.M. and Wasan, E.K. (2017). An Overview of Chitosan Nanoparticles and Its Application in Non-Parenteral Drug Delivery. Pharmaceutics, **9**, 53.
24. Katas, H., Raja, M. A. G., & Lam, K. L. (2013). Development of Chitosan Nanoparticles as a Stable Drug Delivery System for Protein/siRNA. International Journal of Biomaterials, 1–9.
25. Abdul Ghafoor Raja, M., Katas, H., & Jing Wen, T. (2015). Stability, Intracellular Delivery, and Release of siRNA from Chitosan Nanoparticles Using Different Cross-Linkers. PLOS ONE, **10**(6), e0128963.
26. Hassan, U. A., Hussein, M. Z., Alitheen, N., Yahya Ariff, S. A., & Masarudin, M. J. (2018). In vitro cellular localization and efficient accumulation of fluorescently tagged biomaterials from monodispersed chitosan nanoparticles for elucidation of controlled release pathways for drug delivery systems. International Journal of Nanomedicine, **13**, 5075–5095.
27. Chiesa, E., Dorati, R., Conti, B., Modena, T., Cova, E., Meloni, F., & Genta, I. (2018). Hyaluronic Acid-Decorated Chitosan Nanoparticles for CD44-Targeted Delivery of Everolimus. International Journal of Molecular Sciences, **19**(8), 2310.